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CONTRACTING ORGANIZATION: Texas A&M University
College Station, TX 77845

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This application is designed to impact three of the overarching challenges identified for this award type: (i) revolutionary treatment regimes replacing drugs that have life-threatening toxicities with safe and effective interventions; (ii) elimination of mortality associated with metastatic breast cancer; and, (iii) distinguishing aggressive breast cancer from indolent forms, to avoid over-diagnosis and treatment. Overall, this work addresses issues that relate to: (i) revolutionary treatment regimes replacing drugs that have life-threatening toxicities with safe and effective interventions; (ii) elimination of mortality associated with metastatic breast cancer; and, (iii) distinguishing aggressive breast cancer from indolent forms, to avoid over-diagnosis and treatment.

15. SUBJECT TERMS

Nothing listed

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Theranostics Targeting Metastatic Breast Cancer

A. Introduction (1paragraph)

The overall goal of this proposal is to prepare $TrkC^+$-targeted fluorescence/PET/PDT for breast cancer. For this we use a $TrkC^+$ targeting fragment (parts of structures colored in blue throughout this report) and compare them with isomeric compounds that do not target $TrkC^+$ (green). Last year we reported the synthesis aza-BODIPY **1**. This compound was designed for optical imaging of $TrkC^+$ tumors; this year we did that in a 4T1 mouse model, and published the results. Agent **1** accumulated well in tumors, but was not very bright perhaps due to insolubility aggregation effects. We had also proposed to make compound **2** for positron emission tomography (PET) and photodynamic therapy (PDT). After much effort, we made **2** then realized the reason that was so difficult was due to compound instability, specifically the compound loses iodine easily. Instability means **2** is not useful for imaging or therapy. Consequently, just as indicated as a back-up plan last year, we switched focus to the more water-soluble cyanine-based systems (here, **3** - **7**). These are more soluble, brighter, and less prone to aggregation. So far, dyes **3** and **4** have been tested *in vivo*, but they accumulated in the kidneys and cleared quickly with little imaging of the tumor. To address this issue, we have prepared other systems with more lipophilic cyanines (*eg* **5** - **7**), and plan to make other systems with albumin binders to increase retention (based on a literature precedent); all these compounds are ready to be tested *in vivo*. It is necessary to find cyanines that sensitize production of singlet oxygen to realize the overall goal of this study: fluorescent/PET/PDT theranostics. We discovered a brominated cyanine core that does indeed act as a singlet oxygen sensitizer, and compounds **5** – **7** are designed to exploit this. Finally, we prepared the targeted PET agent (**8**), and the targeted maytensin (**9**) mentioned in our previous report (also awaiting *in vivo* studies). The PET agent **8** was tested *in vivo* in a 4T1 mouse model; it had good stability (no 18-fluoride in bone) but cleared quickly. Modifications of this are suggested for year 3. Overall, we are extremely optimistic of reaching our goal for a $TrkC^+$-targeted fluorescence/PET/PDT theranostic for breast cancer.

B. Keywords (limit to 20 words)

reagents for histology of $TrkC^+$ tumors • photodynamic therapy (PDT) • positron emission tomography (PDT)

C. Accomplishments

What were the major goals of the project?

1 Design and synthesis of second-generation fluorescent, PDT and PET/PDT agents *that absorb >700 nm*, bind TrkC, are localized in $TrkC^+$ cells, generate singlet oxygen under conditions for PDT, and have $TrkC^+$ selective photocytoxicities.

begins in year 1 and continues throughout grant period (about 40 % of total work required achieved this year)

2 Validation of a fluorescent form of one of these agents in histochemistry for diagnosis of patients with $TrkC^+$-expressing tumors.

year 2 and then continues throughout grant period

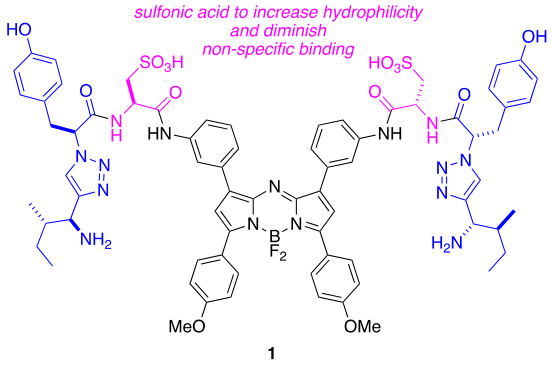
3 Validation of the iodinated second-generation agent PET imaging human breast cancer tumors in mice, and ablation of these tumors via PDT. This study will involve determination of toxicity *in vivo*, pharmaco-kinetics and -dynamics (using PET) to ascertain distribution and clearance of the labels.

only in years 2 and 3

What was accomplished under these goals?

Optical Imaging With Compound **1**

Compound **1** (and the non-targeting, isomeric control, not shown) were tested in the *in vivo* mouse 4T1 model, and the results are summarized below. This study was successful, good accumulation in the tumor was obtained.



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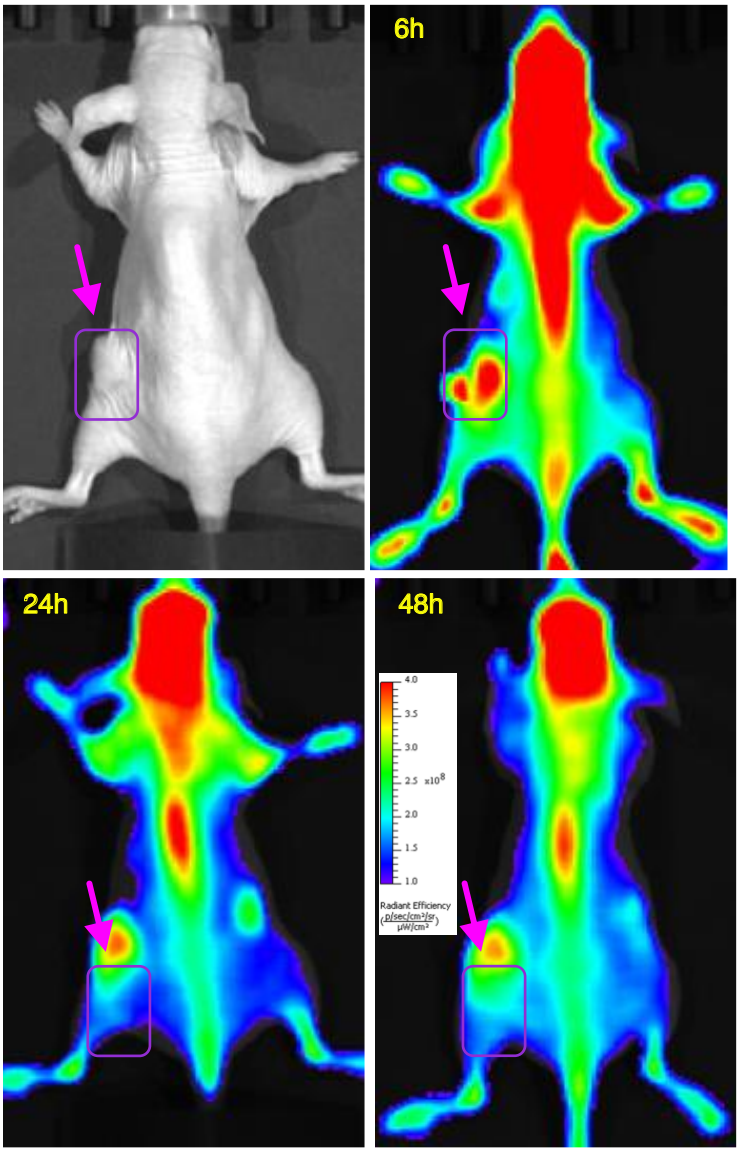


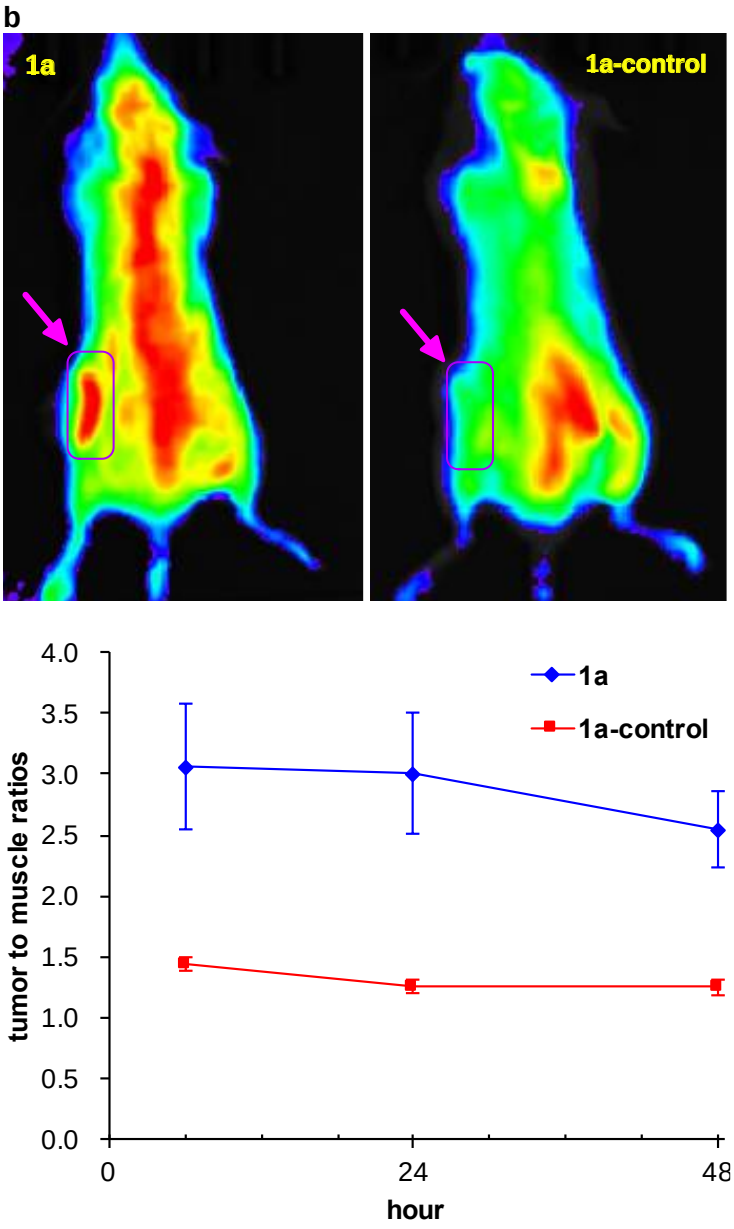
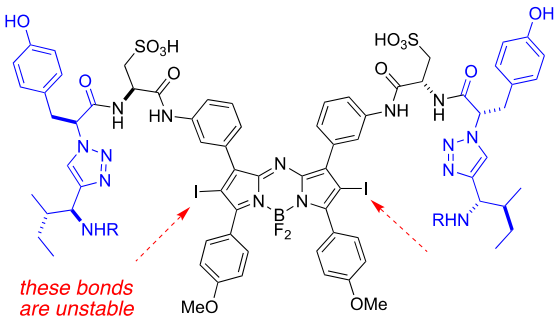


Figure 1. **a** *In vivo* NIR fluorescence images (at 6 h, 24 h, and 48 h p.i.) of 4T1 tumor-bearing mice injected (iv) with **1a**. Arrows indicate the tumors. **b** Representative NIR fluorescent images of mice injected with **1** (left) or **1-control** compound at 48h p.i. (right). The arrows indicate the tumors (top); a region of interest (ROI) study of tumor/normal tissue (muscle) ratio of **1a** and **1-control** in the imaging groups (n = 3).

The corresponding iodinated aza-BODIPY **2** was unstable (see below) hence this aza-BODIPY approach could not lead to the integrated fluorescent/PET/PDT theranostic that we are determined to prepare. Consequently, this approach was abandoned in favor of ones featuring cyanine dyes. We felt the superior solubility of select cyanine dyes would reduce aggregation, and increase brightness in optical imaging studies. Moreover, it is easier to design cyanines that absorb over 750 nm than it is for aza-BODIPYs.

Compound **2** Intended For PET and PDT Imaging

Compound **2** (and the corresponding control). These syntheses are about 10 steps, and the final product kept decomposing making us think the reactions to form it had failed. Agent **2** is *not* a good approach to the theranostic, and we regret spending so much time on this.

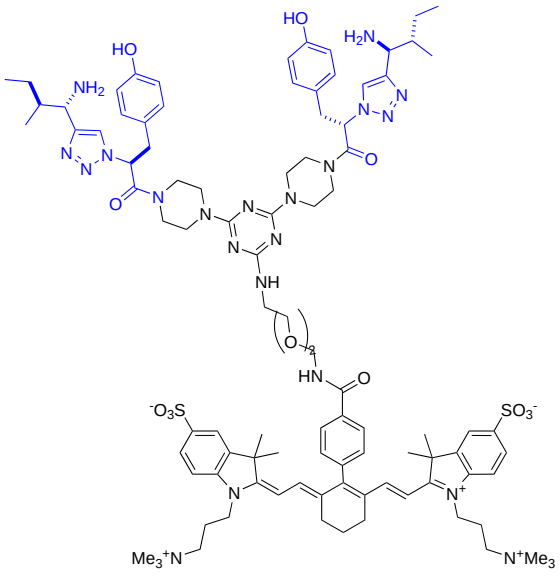


2

Change of strategy to the cyanine dyes was important; and this has been much more fruitful, though not without set-backs.

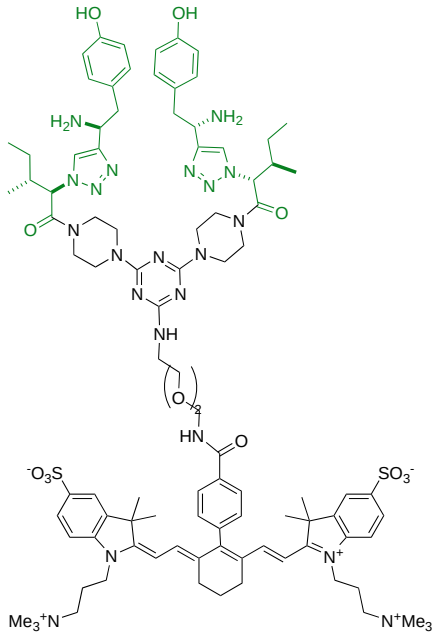
Investigation Of Cyanine Systems **3** and **4** For Optical Imaging

We prepared systems **3** (and the control indicated), and obtained photphysical properties for these agents. These cyanines absorb around 760 nm and fluoresce with a maximum of around 790 nm, which is ideal for optical imaging *in vivo*. In cell studies on a high-power fluorescent microscope we can see that the targeted dye **3** has a higher affinity to NIH 3T3 TrkC$^+$ transfectant cells than the control. However, because of filter issues, we are currently not able to image live cells treated with these agents on our confocal apparatus (we are trying to vary conditions to correct this). In histology, however, **3** stains metastatic breast cancer much more efficiently than the control **3-control**.



IY-IY-Targeted Cyanine

3



YI-YI (Non-targeted Cyanine)

3-control

Optical imaging of these compounds was performed in the 4T1 murine model, and in another TrkC^{+}, Hs578t implanted into nude mice (better image since these mice have no hair). The data (Fig 1) show these agents were not retained well; they accumulated in the kidneys and were cleared rapidly.

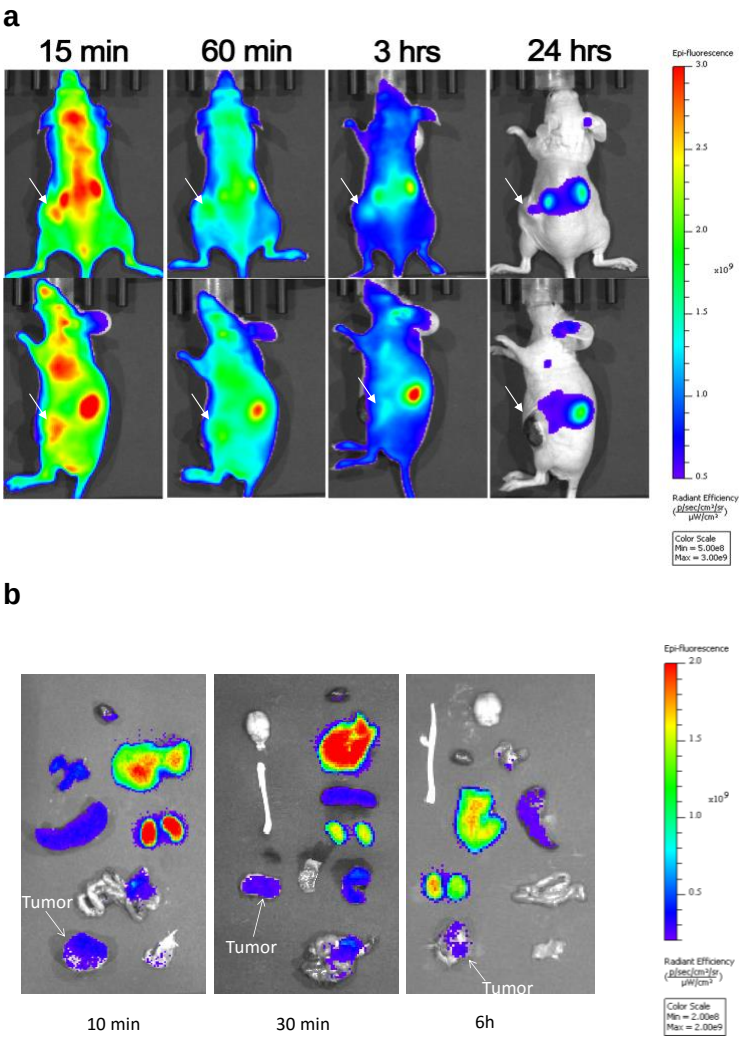
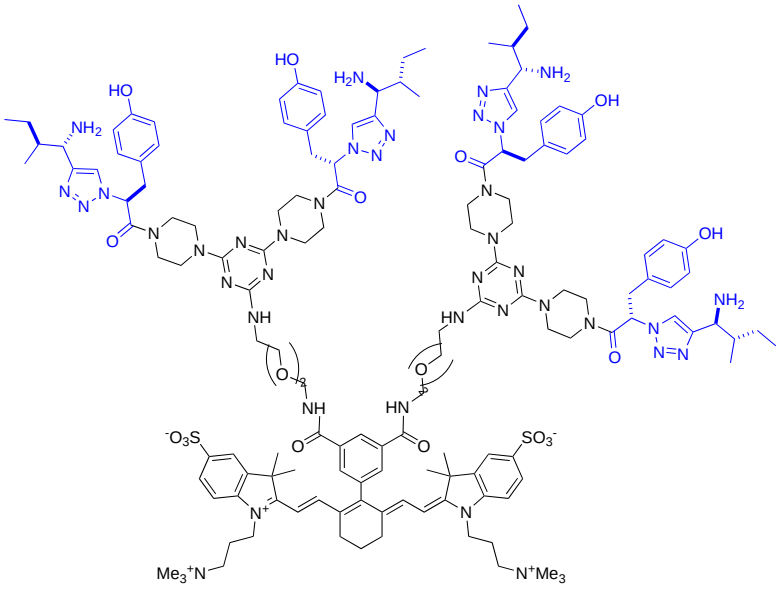
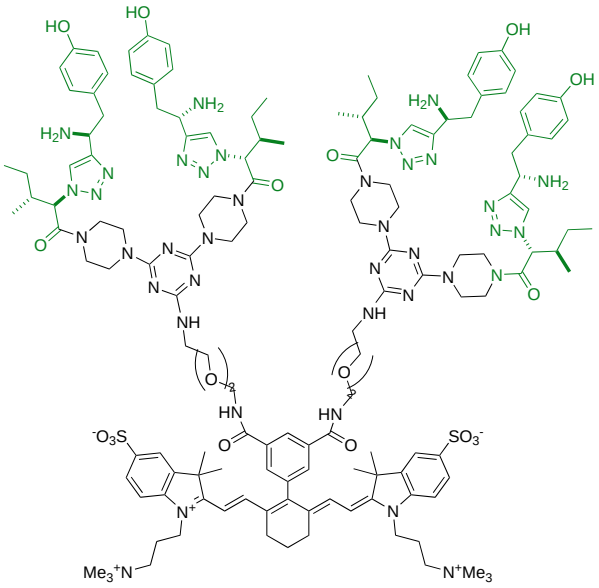


Figure 1. **a** Optical imaging of probe KB2251 in 4T1 breast cancer bearing mice (n=3). Murine 4T1 cancer cells with TrkC expression were inoculated into nu/nu mouse to create breast cancer animal model. Probe KB2551 (5 nmol/mouse) was injected via tail vein, and in vivo optical imaging was captured with an IVIS200 imaging system and quantified by Living Imaging software (Xenogen, Alameda, CA). Images were acquired at 15mins, 60mins, 3 h, and 24 h postinjection (p.i.). Excitation and emission filters were set at 745 and 800 nm respectively as suggested by the system for image acquisition. Tumors are indicated by arrowheads. Tumor uptake was clearly visualized at early time point but the probe is very highly hydrophilic and is washed out quickly. High kidney uptake at early time point and rapidly decreased demonstrating a fast renal excretion. From this result, new probe with an Albumin binder is designed for increasing the circulation time, the next generation compound will be prepared by Dr. Burgess's lab following the in vitro and in vivo validation. **b** Biodistribution study of KB2251 compound in 4T1 breast cancer bearing mice. Tumors and other major organs (i.e., liver, spleen, kidney, heart, lung, brain, spinal cord and intestines) were dissected intact upon immediately sacrificing the mice at 10min, 30min and 6h. White arrow marks the tumor.



IY-IY (Targeted Cyanine)

4



YI-YI (Non-targeted Cyanine)

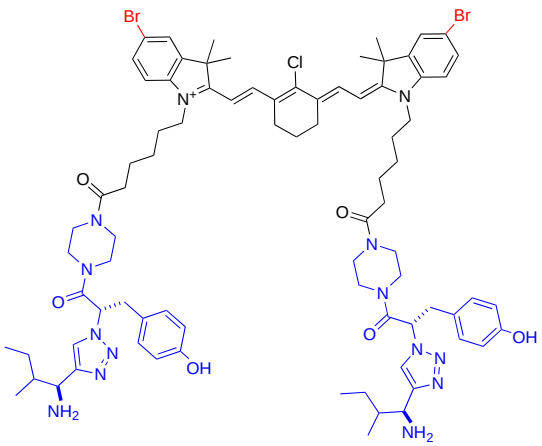
4-control

We also prepared compounds **4** and **4-control** suspecting that these molecules having *two* IY-IY binding groups would have an even higher affinity for cells expressing TrkC$^+$. In the event, this appeared to be true in live cell staining. However, for reasons we currently do not understand, *in vivo* these compounds were *less* visible in nearIR imaging.

Our conclusions from these studies are that the dye should be made more lipophilic or should be modified to incorporate an albumin binding molecule (based on literature precedent).

More Lipophilic Cyanine Systems That Should Be Cleared More Slowly

Compounds **5** and **6** were designed to have cyanine dyes that are less hydrophilic than **3** and **4**. So far we have determined that these systems do indeed bind NIH3T3 TrkC$^+$ transfectant cells, and metastatic breast cancer in histology experiments (only stained metastatic breast cancer {12 samples} and not normal breast tissue {12 samples}. The water-solubilities of these systems were also determined (**5** is 45 μM in 0.5 % DMSO in 1 M PBS, 7.24 pH; **6** is far more soluble). We have not yet been able to determine a K_d for these compounds binding the TrkC$^+$ receptor because there is no obvious “blocking group” for use in cells studies; however, we continue to search for a work-around to this problem. In photocytoxicity experiments compound **5** was not cytotoxic in the dark to NIH3T3 TrkC$^+$ at 40 μM, but was highly toxic when illuminated at 780 nm. Compounds **5** and **6** have the potential to be near-IR imaging agents and PDT therapeutics *in vivo* (see below). They will be tested in this upcoming research year.



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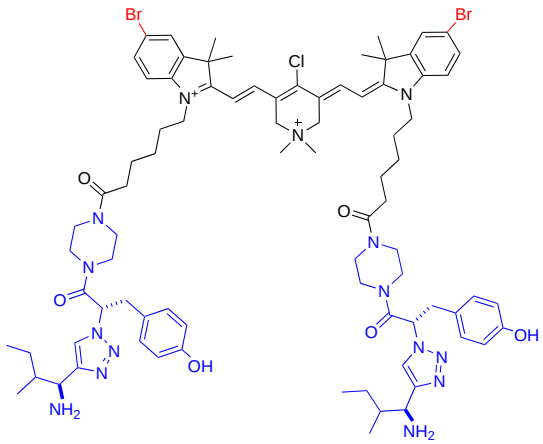


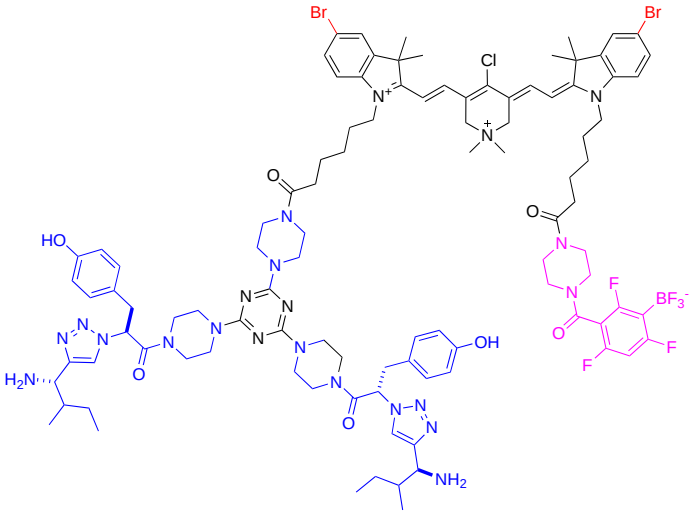
6

Cyanine Dyes In PDT

It transpires that relatively little is known about cyanine dyes in PDT; there have been only a few studies. We found that the parent dibromide cyanines from which **5** and **6** are made are good PDT agents. While these experiments were in progress another group reported PDT studies on very similar {untargeted} diiodide systems. The *in vivo* work alluded to above should be extremely interesting; it may be the first targeted study with cyanine dye sensitizers.

Potential Fluorescent/PET/PDT Theranostic **7**

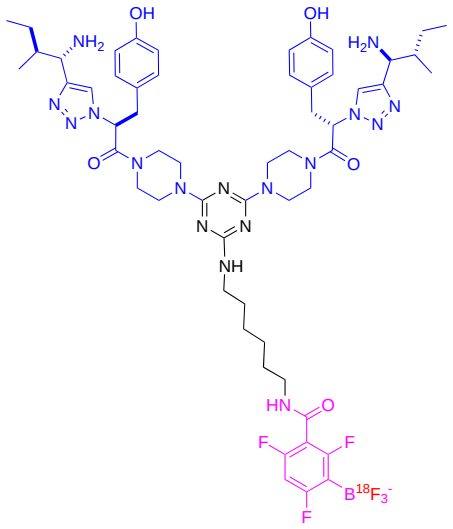
We have also prepared compound **7** which could become our lead theranostic. This structure has the TrkC^{+} targeting group (blue), a Perrin ^{18}F capture unit (purple) and a PDT active cyanine backbone: all the components for a fluorescent/PET/PED theranostic. Currently we are scaling up the synthesis of this molecule, for photophysical measurements, cellular assays particularly light/dark cytotoxicity, then *in vivo* studies.



7

Targeted PET Label **8**

Our objective was to perform at least one set of PET experiments in year 2, consequently we made and tested the TrkC^{+} targeted PET agent **8**. Exchange of ^{19}F with ^{18}F was performed on this system with a 6 – 8 % radiochemical yield. Unfortunately, the pharmacokinetic properties of this agent were unfavorable because it cleared too rapidly. However, it dd not release ^{18}F *in vivo*; we know this because the label did not accumulate in the bone.



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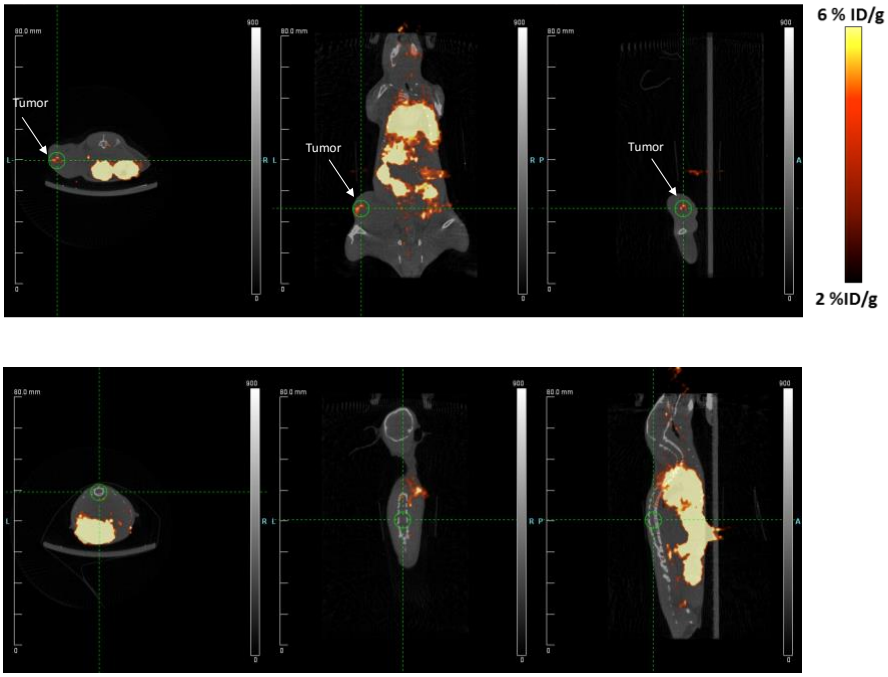
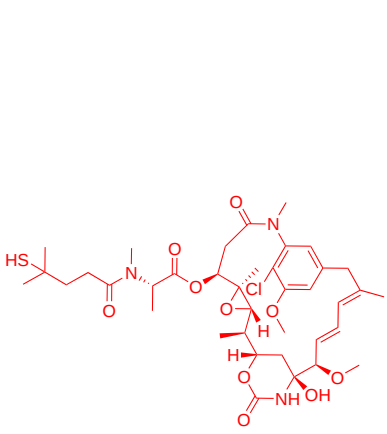


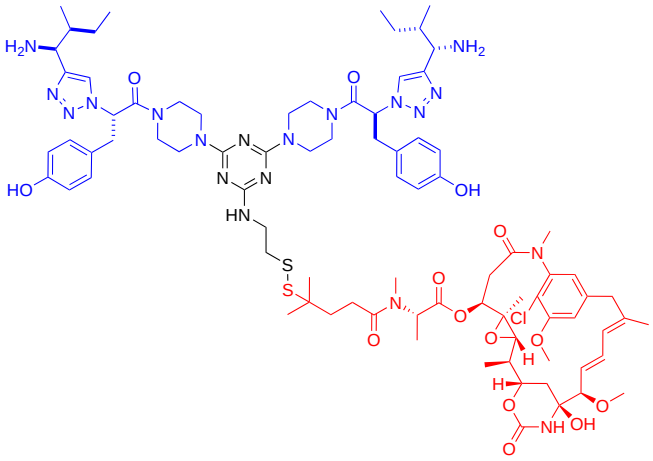
Figure 3. Small-animal PET/CT imaging of U-87 tumor-bearing mice. Small-animal PET/CT scans of 4T1 tumor–bearing mice injected intravenously with approximately 3.7 MBq of F-18 KB2265 at 10mins p.i. Tumors are indicated by arrowheads (top). There is no uptake in the bone and spinal cord indicating good in vivo stability of the F-18 KB2265 (bottom).

Targeted Maytensinoid Therapeutic **9**

Finally, in collaboration with a biotech company who donated the maytansinoid **DM4**, in out labs we prepared the targeted conjugate **8** equipped with a disulfide linker designed to be cleaved in the cytosol. Cell studies with agent **9** in comparison with **9-control** indicate there is slightly improved cellular therapeutic index. However, we expect the improvements to therapeutic index *in vivo* to be more profound because of pharmacokinetic effects that cannot be replicated in cell culture. This compound awaits *in vivo* studies.



maytansinoid DM4



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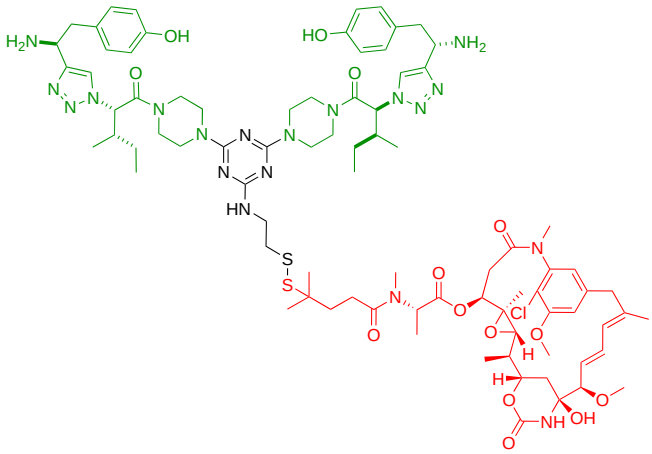

9-control

What opportunities for training and professional development has the project provided?

This funding has been used to support postdoctoral fellow Jaya Shrestha, and graduate students Usama Syed, Bosheng Zhou, and Daniel Jiang.

How were the results disseminated to communities of interest?

These papers acknowledge support from this grant.

310 Small Molecule Inhibitors of the PCSK9-LDLR Interaction, J. Taechalertpaisarn, B. Zhao, X. Liang, K. Burgesss, *JACS*, 2017, under revision.

309 How Relevant is Secondary Structure Mimicry in Design of Small Molecules to Disrupt Protein-protein Interfaces?, J. Taechalertpaisarn, M. Arancillo, C.-M. Lin, R.-L. Lyu, Z. Jiang, L. M. Perez, T. R. Ioerger, K. Burgess, *Chem. Sci.* 2017, submitted.

308 An Agent for Optical Imaging of TrkC-Expressing, Metastatic Breast Cancer, A. Kamkaew, F. Li, Z. Li, K. Burgess, *Med. Chem. Commun.* **8**, 1946-1952, 2017, (DOI: 10.1039/c7md00328e)

307 Interplay of Stereochemistry, Conformational Rigidity, and Ease of Synthesis for 13-Membered Cyclic Peptidomimetics Containing APC Residues, D. Xin, A. Jeffries, K. Burgess, *ACS Comb. Sci.*, 2017, 414-421.

306 Novel Small Molecule Probes for Metastatic Melanoma, K. Burgess, A. Kamkaew, N. Fu, W. Cai, *ACS Med. Chem. Lett.*, 2016, **8**, 179-184. (DOI: 10.1021/acsmedchemlett.6b00368)

305 Heterogeneous Phase Transfer Catalysis in Solid Phase Syntheses of Anth-Cyclic Tetrapeptides, D. Xin, K.-Y. Wong, K. Burgess, *J. Org. Chem.*, 2016, **81**, 8077-8081.

304 Small Molecules for Active Targeting in Cancer, C. S. Kue, A. Kamkaew, K. Burgess, L. V. Kiew, L. Y. Chung, H. B. Lee, *Med. Res. Rev.,* 2016, **36**, 494-575. (DOI:10.1002/med.21387)

The PI gave a seminar “Active Targeting In Cancer” at the following venues:

Active Targeting of TrkC In Cancer, University of Regensburg, GERMANY, June 2017.

Active Targeting of Cancer Cells, City University of Hong Kong, November 2016.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period we will:

(i) initiate *in vivo* studies (PDT and PET) on some of the compounds already prepared; and,

(ii) continue the synthetic studies to produce agents with PDT characteristics.

We are absolutely determined to realize the overall goal of this proposal: a theranostic that can be used for diagnosis via histology, imaging via PET, and therapy via PDT.

D. Impact

What was the impact on the development of the principle(s) of the project?

At this early stage, the impact is mostly on improvement of the chemical design of the agents being developed. In this grant period we have begun to make some novel cyanine systems with improved solubility characteristics and suitable for targeted PDT.

What was the impact on other disciplines?

In the broader sense we hope to prepare fluorescent and PET imaging agents that can differentiate between TrkA – C *in vivo*. This will be useful as a probe in cell biology, *in vivo*, and potentially (beynd the scope of this proposal) in clinical imaging.

What was the impact on technology transfer?

A patent application covering the IY-IY targeting groups is now approved.

Dipeptide Mimics, Libraries Containing Tow Dipeptide Mimics With A Third Group, And Methods Of Production Thereof, Kevin Burgess, US 9,562,023 B2, issued Feb 7, 2017.

What was the impact on society beyond science and technology?

Too early to impact society outside science, but eventually the goal of this work is to produce a lead compound that will be iteratively improved to form a "theranostic" for diagnosis, imaging, and therapy.

E. Changes/Problems

Changes in approach and reasons for change

The goal is the same, the particular agents we are preparing to realize the goal are different as detailed above.

Actual or anticipated problems or delays and actions or plans to resolve them

Problems are nearly always encountered in chemical syntheses, but we have anticipated the obvious weak links and constantly consider alternative routes. We have several agents prepared as described above, and more possibilities that we did not detail here.

We see more of opportunities to use cyanine dyes that, remarkably, seem to accumulate in all types of cancer cells. Thus a back-up plan is to use these without any targeting group as theranostics.

Changes that had a significant impact on expenditures

We made a request to re-budget some funds from salaries to equipment to buy an HPLC for preparative isolation of the target molecules. This request was granted, the machine is scheduled for delivery at the end of November and will accelerate progress.

The budget as originally requested emphasized chemistry in years 1 and 2, less in year 3. This is because we knew chemical synthesis tends to be the slow step. The synthetic efforts are still crucial to the overall success of this project. It is therefore fortunate that there is a balance carry over into the next year, this will be used to support the synthesis efforts, and possibly to initiate some *in vivo* studies at TAMU (see below).

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None, except that NO ANIMAL STUDIES HAVE BEEN PERFORMED AT TAMU. We had budgeted a small amount for Dr Deanne Wallis at TAMU to perform a small toxicology study, but Dr Wallis left TAMU at the end of last year. All the *in vivo* work has therefore been performed at Methodist in Dr Li's group.

Going forward, since we are generating many agents to test *in vivo*, there is a possibility that some studies will be performed at TAMU. Dr Li's speciality is optical and PET imaging, and she will continue to do that. However, if we can recruit a person with appropriate expertise at TAMU, then some of the PDT studies might be attempted there. IF THIS CHANGE IS MADE, APPROPRIATE APPROVAL WILL BE OBTAINED BEFORE ANY EXPERIMENTS BEGIN.

Significant changes in use of care of human subjects

n/a

Significant changed in use of care of vertebrate animals

none

Significant changes in use of biohazards and/or select agents

none

F. Products

Publications, conference papers, and presentations

Please see list of presentations above.

Websites or other Internet sites

none

Technologies or techniques

See above.

Inventions, patent application, and/or licenses

See patent issued cited above

Other Products

none

G. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Zhengyang (Daniel) Jiang, gradate student at TAMU, 50% time, 1 year.
Syed Usama, graduate student at TAMU 50% time for 7 months, 25% time for 1 month, 8 months total work.
Bosheng Zhao, graduate student at TAMU 50% time for 6 months, 25% time for 1 month, 7 months total work.
Dr Chen-Ming Lin, postdoctoral associate, 5 months effort at 100 %.
Dr Jay Shrestha, postdoctoral associate, 100% time for 8 months.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel?

no

What other organizations were in involved as partners?

Dr Li, co-PI at Methodist is about to begin.

Special Reporting Requirements

none

H. Appendices (attached journal articles, reprints, CV, patent applications)

Reprints of our most important papers, and of the issued patent are attached.

US009562023B2

(12) **United States Patent**
Burgess

(10) **Patent No.: US 9,562,023 B2**
(45) **Date of Patent: Feb. 7, 2017**

(54) **DIPEPTIDE MIMICS, LIBRARIES COMBINING TWO DIPEPTIDE MIMICS WITH A THIRD GROUP, AND METHODS FOR PRODUCTION THEREOF**

(75) Inventor: **Kevin Burgess**, College Station, TX (US)

(73) Assignee: **Kevin Burgess**, College Station, TX (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/418,917**

(22) Filed: **Mar. 13, 2012**

(65) **Prior Publication Data**

US 2012/0232268 A1 Sep. 13, 2012

Related U.S. Application Data

(63) Continuation of application No. 12/181,168, filed on Jul. 28, 2008.

(60) Provisional application No. 60/952,149, filed on Jul. 26, 2007.

(51) **Int. Cl.**

C07D 403/14	(2006.01)
C07D 249/04	(2006.01)
C07D 241/08	(2006.01)
C07D 271/10	(2006.01)
C07D 273/00	(2006.01)
C07D 401/04	(2006.01)
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None

See application file for complete search history.

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(57) **ABSTRACT**

Monovalent compounds having moieties comprising at least one amino acid side chain are bound to a core molecule, which also comprises a nucleophilic moiety bound to said core molecule. Monovalent compounds also comprise a macrocyclic ring, a nucleophilic moiety, and a spacer group. Monovalent compounds may be combined into bivalent and trivalent compounds, some of which may have a labeling tag. Methods of production of bivalent compounds and contemplated uses thereof are disclosed.

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An agent for optical imaging of TrkC-expressing, breast cancer†

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Tropomyosin receptor kinases receptor C is expressed at high levels on the surface of tumors from metastatic breast cancer, metastatic melanoma, glioblastoma, and neuroblastoma. Previous studies have shown synthetic TrkC ligands bearing agents for photodynamic therapy could be used to completely ablate 4T1 metastatic breast tumors and suppress metastatic spread *in vivo*. Modification of these probes (**A** in the text) to make them suitable for near infrared optical imaging *in vivo* would require a substantial increase in molecular mass (and hence increased vulnerability to undesirable absorption, metabolism and immunogenicity effects), or significant changes to the probe design which might compromise binding to TrkC in histochemical studies and on live cells. The research featured here was undertaken to investigate if the second strategy could be achieved without compromising binding to TrkC-expressing tissues. Specifically, an "aza-BODIPY" probe was synthesized to replace a spacer fragment in the original probe **A**. In the event, this new probe design (**1a** in the text) binds TrkC$^+$ breast cancer in live cell cultures, in histochemical studies and in an *in vivo* murine model. Probe **1a** binds TrkC$^+$ tissues with good contrast with respect to healthy tissues, and much more strongly than an isomeric, non-TrkC binding, probe (**1b**) prepared as a negative control.

Introduction

Tropomyosin receptor kinases (Trk's) are cell-surface receptors that bind the neurotrophin growth hormones.[1] Docking neurotrophin-3 (NT3) with TrkC, for instance, triggers intracellular phosphorylation then a network of cell signaling processes that lead to cell growth and differentiation.[2,3] Overexpression of Trk receptors is associated with several forms of cancer, including neuroblastoma,[4–16] glioblastoma,[14,17–21] metastatic breast cancer[22–28] and metastatic melanoma.[29–37] Consequently, functionalized Trk ligands may be prepared as possible imaging agents for cancer.[28,38–40] Synthetic TrkB-[41] and pan-Trk-binding agents[16] functionalized with radionuclei have been explored for imaging with positron emission tomography (PET), but, to the best of our knowledge, no synthetic imaging agents have been reported for selective optical-imaging of TrkC *in vivo*.

Prior research from these laboratories featured the TrkC-homing ligand in structure **A**. Probe **A** was shown to be internalized by TrkC-expressing cells and it could also be used as a staining agent for metastatic breast cancer and melanoma tissue, both of which are associated with TrkC overexpression. Moreover, one 10 mg kg^{-1} dose of **A** coupled to a BODIPY-based PDT agent was shown to almost completely ablate a 4T1 primary tumor, and suppress metastatic spread.[42]

Probe **A** is not ideal for optical imaging because it absorbs light optimally at a wavelength (around 500 nm) that is too short for efficient penetration of more than a few mm in tissue.[43,44] To circumvent this, the most obvious solution would be to prepare similar molecules with a near-IR-absorbing probe directly replacing the BODIPY. However, modifications to fluors to make them near-IR absorbing invariably increase their molecular size; this is undesirable because larger structures tend to be more vulnerable to undesirable absorption, metabolism, and excretion effects. We reasoned that one way to reconcile these opposing design criteria is to replace the black part of structure **A** with a near-IR fluor giving the generic structures **B** (Fig. 1). Designs **B** eliminate the triazine core which serves no purpose other than as a scaffold-spacer. One possibility would be to replace it with an aza-BODIPY dye; these dyes tend to have significantly longer absorption and emission wavelength maxima than their BODIPY analogs.[45–47]

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† Electronic supplementary information (ESI) available: Scheme and full details of compounds syntheses and characterizations, cell toxicity of **1a** and TrkC antibody stained on breast tissues. See DOI: 10.1039/c7md00328e

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Novel Small Molecule Probes for Metastatic Melanoma

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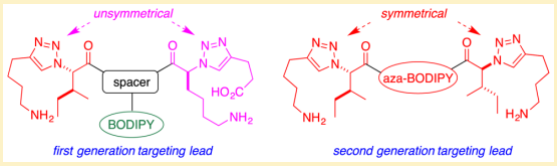
Supporting Information

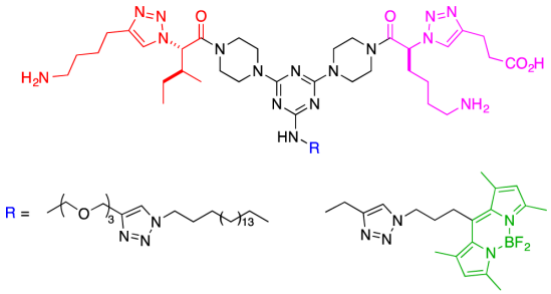
ABSTRACT: Actively targeting probe **1b**, an unsymmetrical bivalent dipeptide mimic, selectively bound melanoma over healthy skin tissue in histological samples from patients and Sinclair swine. Modifications to **1b** gave agents **2**−**4** that contain a near-IR aza-BODIPY fluor. Contrary to our expectations, symmetrical probe **3** gave the highest melanoma-to-healthy skin selectivity in histochemistry and experiments with live cells; this was surprising because **2**, not **3**, is unsymmetrical like the original lead **1**. Optical imaging of **3** in a mouse melanoma model failed to show tumor accumulation *in vivo*, but the probe did selectively accumulate in the tumor (some in lung and less in the liver) as proven by analysis of the organs post mortem.



KEYWORDS: *Targeting, cancer, melanoma, small molecule ligand*

Molecular fragments that bind receptors selectively overexpressed on tumor cell surfaces can be useful for active targeting.[1] Active targeting is distinct from strategies designed to perturb specific biochemical pathways upregulated in cancer, e.g. involving kinases or proteases. Small molecules that are commonly used for active targeting include some vitamins (e.g., folic acid,[2−5] biotin,[6] and cobalamin[7,8]), RGD peptidomimetics,[9−13] a few carbonic anhydrase ligands,[14−17] and mimics of the prostate specific antigen,[18,19] but relatively little else. This is limiting because not all tumor types overexpress the corresponding receptors at usable cell surface copy-numbers, and some of these ligands have suboptimal properties for targeting entities.[20]

To facilitate discovery of novel ligands for targeting, we reported[21] the method shown in Figure 1 to identify small molecules that bind unknown receptors selectively expressed on the surface of cancer cells. In that method, a set of monovalent dipeptide mimics were designed and prepared,[22−25] with a bias toward two side-chain pharmacophores that correspond to the most common amino acids found at protein−protein interfaces (Trp, Arg, Tyr, Lys, Glu, then Ser, Asn, Leu).[26] Our hypothesis is that these small molecules mimic side-chain orientations on various dipeptides that have a relatively high tendency to bind other proteins. Amino acid side-chains are important because they tend to dominate the thermodynamics of protein−protein interactions.[26]



These monovalent molecules were assembled into bivalent ones **1a** bearing a long hydrophobic chain substituent as shown above. Only *n* monovalent compounds are needed to make $n(n + 1)/2$ bivalent ones, so large libraries can be made from a small number of building blocks.[27] In a one-compound-per-well format, each bivalent molecule was allowed to associate with a liposome via capture of the hydrophobic side-chain into the surface bilayer. All the liposomes used in these experiments carried the plasmid encoding luciferase. Cells will fuse with liposomes at a certain rate, but this happens faster if the liposome-supported bivalent compounds bind a cell-surface receptor and mediate internalization. The degree of import after

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